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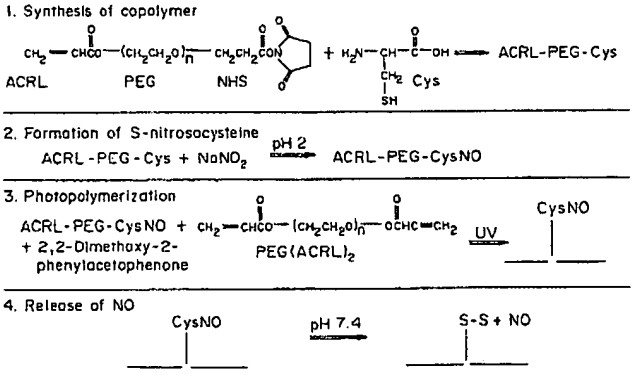
(54) Title: NITRIC OXIDE-PRODUCING HYDROGEL MATERIALS



(57) Abstract: Hydrogels releasing or producing NO, most preferably polymerizable biodegradable hydrogels capable of releasing physiological amounts of NO for prolonged periods of time, are applied to sites on or in a patient in need of treatment thereof for disorders such as restenosis, thrombosis, asthma, wound healing, arthritis, penile erectile dysfunction or other conditions where NO plays a significant role. The polymeric materials can be formed into films, coatings, or microparticles for application to medical devices, such as stents, vascular grafts and catheters. The polymeric materials can also be applied directly to biological tissues and can be polymerized in situ. The hydrogels are formed of macromers, which preferably include biodegradable regions, and have bound thereto groups that are released in situ to elevate or otherwise modulate NO levels at the site where treatment is needed. The macromers can form a homo or hetero-dispersion or solution, which is polymerized to form a hydrogel material, that in the latter case can be a semi-interpenetrating network or interpenetrating network. Compounds to be released can be physically entrapped, covalently or ionically bound to macromer, or actually form a part of the polymeric material. The hydrogel can be formed by ionic and/or covalent crosslinking. Other active agents, including therapeutic, prophylactic, or diagnostic agents, can also be included within the polymeric material.

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NITRIC OXIDE-PRODUCING HYDROGEL MATERIALS

FIELD OF THE INVENTION

The present invention relates to polymerizable hydrogel materials that produce physiologically relevant amounts of nitric oxide (NO).

BACKGROUND OF THE INVENTION

Endothelial cells, normally present as a monolayer in the intimal layer of the arterial wall, are believed to play an important role in the regulation of smooth muscle cell (SMC) proliferation *in vivo*. Endothelial cells are seriously disrupted by most forms of vascular injury, including that caused by percutaneous transluminal coronary angioplasty and similar procedures. Approximately 35-50% of patients treated by percutaneous transluminal coronary angioplasty experience clinically significant renarrowing of the artery, or restenosis, within six months of the initial treatment. Restenosis is due, at least in part, to migration and proliferation of smooth muscle cells in the arterial wall along with increases in secretion of matrix proteins to form an obstructive neointimal layer within the arterial wall. Similar issues limit the performance of vascular grafts. The processes that regulate arterial wound healing following vascular injury, such as that caused by angioplasty, are as yet poorly understood, but are believed to involve a complex cascade of blood and vessel wall-derived factors.

Numerous factors that stimulate intimal thickening and restenosis have been identified through administration of exogenous proteins, genetic alteration of cells, or through the blockade of certain signals using antibodies or other specific growth factor inhibitors. These smooth muscle cell mitogens and chemoattractants derive from both the blood or thrombus formation and from the vessel wall itself. Endothelial cells produce a number of substances

known to down-regulate smooth muscle cell proliferation, including heparin sulfate, prostacyclin (PG12), and NO.

NO is an endothelium-derived target molecule useful for the prevention of restenosis because, in addition to limiting the proliferation of smooth muscle cells (Garg et al., (1989) *J. Clin. Invest.*, 83:1774-7), NO reduces platelet aggregation (de Graaf et al., (1992) *Circulation*, 85:2284-90; Radomski et al., (1987) *Br. J. Pharmacol.*, 92:181-7), increases endothelial cell proliferation (Ziche et al., (1993) *Biochem. Biophys. Res. Comm.*, 192:1198-1203), and attenuates leukocyte adhesion (Lefer et al., (1993) *Circulation*, 88:2337-50), all of which are highly desirable for the reduction of intimal thickening and restenosis (Loscalzo, (1996) *Clin. Appl. Thromb. Hemostas.*, 2:7-10). Because of the complexity of the restenotic process, approaches that act upon multiple targets are the most likely to be successful.

The mechanisms whereby NO affects these multiple responses are not fully understood as yet, but it is known that NO activates soluble guanylate cyclase by binding to its heme moiety, thereby elevating the levels of cyclic guanosine monophosphate (cGMP), an intracellular second messenger with multiple cellular effects (Moro et al., (1996) *Proc. Natl. Acad. Sci. USA*, 93:1480-5). The effects of NO can often be mimicked by the administration of cGMP or more stable derivatives of cGMP (Garg et al., (1989) *J. Clin. Invest.*, 83:1774-7). In addition, NO has been found to inhibit ribonucleotide reductase, an enzyme that converts ribonucleotides into deoxy ribonucleotides, thus significantly impacting DNA synthesis (Lepoivre et al., (1991) *Biochem. Biophys. Res. Comm.*, 179:442-8; Kwon et al., (1991) *J. Exp. Med.*, 174:761-7), as well as several enzymes involved in cellular respiration (Stuehr et al., (1989) *J. Exp. Med.*, 169:1543-55).

A number of molecules that produce NO under physiological conditions (NO donors) have been identified and evaluated both *in vitro* and *in vivo*. NO donor molecules exert biological effects mimicking those of NO

and include S-nitrosothiols (Diodati et al, (1993) *Thromb. Haem.*, 70:654-8; Lefer et al., (1993) *Circulation*, 88:2337-50; DeMeyer et al., (1995) *J. Cardiovasc. Pharmacol.*, 26:272-9), organic nitrates (Ignarro et al., (1981) *J. Pharmacol. Exp. Ther.*, 218:739-49), and complexes of NO with nucleophiles (Diodati et al., (1993) *Thromb. Haem.*, 70:654-8; Diodati et al., (1993) *J. Cardiovasc. Pharmacol.*, 22:287-92; Maragos et al., (1993) *Cancer Res.*, 53:564-8). Most of these have been low molecular weight molecules that are administered systemically and have short half-lives under physiologic conditions, thus exerting effects upon numerous tissue types with a brief period of activity. In addition, L-arginine is often thought of as a NO donor, as L-arginine is a substrate for NO synthase, and thus administration of L-arginine increases endogenous NO production and elicits responses similar to those caused by NO donors in most cases (Cooke et al., (1992) *J. Clin. Invest.*, 90:1168-72).

The development of NO-releasing polymers containing NO/nucleophile complexes has been reported by Smith et al., (1996) *J. Med. Chem.*, 39:1148-56. These materials were capable of releasing NO for as long as five weeks *in vitro* and were able to limit smooth muscle cell proliferation in culture and to reduce platelet adherence to vascular graft materials in an arterio-venous shunt model. These materials show promise for numerous clinical applications where localized NO production would be desired, such as anti-thrombotic coating materials for catheters, but probably will not be useful for the direct treatment of tissues *in vivo* as these materials suffer from a number of disadvantages. These polymers may be produced as films, powders, or microspheres, but they cannot be formed *in situ* in direct contact with cells and tissues, thus making it difficult to strictly localize NO treatment to a tissue and potentially causing issues with the retention of the polymer at the site of application. The formulation issues will also make local administration during laparoscopic or catheter-based procedures difficult or

impossible. Additionally, biocompatibility of the base polymer is a serious issue for implantable, NO-releasing polymers, especially those intended for long-term use, as inflammatory and thrombotic responses may develop after the cessation of NO release.

With respect to chronic wound healing, approaches that are common today are typically based on simple wound care regimens involving debridement, cleaning, and application of moist dressings (Thomas S, Leigh (1998). WOUND DRESSINGS. WOUNDS: BIOLOGY AND MANAGEMENT, D. Leaper and K. Harding. New York, NY, Oxford University Press). More advanced dressings such as topical gels containing growth factors have resulted in enhanced healing rates in some clinical studies (Wieman T, Smiell J, Su Y. Efficacy and safety of a topical gel formulation of recombinant human platelet-derived growth factor-BB (beclapermin) in patients with nonhealing diabetic ulcers: a phase III randomized, placebo-controlled, double-blind study. *Diabetes Care 1998; 21: 822-827*; Wieman TJ and the Beclapermin Gel Studies Group. Clinical efficacy of Beclapermin (rhPDGF-BB) Gel. *Am J Surg* 1998; 176: 74S-79S; Martinez-de Jesus FR, Morales-Guzman M, Gastaneda M, Perez-Morales A, Garcia-Alsono J, Mendiola-Segura L. Randomized single-blind trial of topical ketanserin for healing acceleration of diabetic foot ulcers. *Arch Med Res 1997; 28: 95-99*), however on the whole these treatments are difficult to apply and are often too expensive for application to large, chronic wounds. Additionally, not all chronic wounds display growth factor deficiencies, and other mechanisms such as rapid degradation by wound proteinases may be involved in the reduction of growth factor levels observed in many chronic wounds. Many chronic wounds are unresponsive to growth factor therapy (Greenhalgh D. The role of growth factors, in wound healing. *J Trauma* 1996; 41: 159-167).

With respect to proliferation of endothelial cells, it has been shown that the presence of NO decreases endothelial cell proliferation. See, for example,

Heller, R., Polack, T., Grabner, R., Till, U. (1999) "Nitric oxide inhibits proliferation of human endothelial cells via a mechanism independent of cGMP", *Atherosclerosis*, 144:49-57; and Sarkar, R., Webb, R.C., Stanley, J.C. (1995) "Nitric oxide inhibition of endothelial cell mitogenesis and proliferation", *Surgery*, 118:274-9. However, these studies utilized very high doses of NO-releasing drugs, which may account for the decreased endothelial cell proliferation. Additionally, previous researchers have found it difficult to seed endothelial cells onto devices: Scott-Burden, T., Tock, C.L., Schwarz, J.J., Casscells, S.W., Engler, D.A. (1996) "Genetically engineered smooth muscle cells as linings to improve the biocompatibility of cardiovascular prostheses", *Circulation*, 94:235-8.

It is believed by the inventors that the development of materials that encourage the proliferation and/or migration of endothelial cells should enhance the growth of endogenous endothelial cells from tissue surrounding an implant onto the implant surface. Therefore, applicants propose that endothelialization of blood-contacting implants, such as stents, grafts, and ventricular assist devices, may significantly improve device performance by decreasing thrombogenicity and smooth muscle cell proliferation.

It would be more efficient if NO releasing compounds or compounds modulating NO levels could be administered solely to the site in need of treatment, and in some cases, reduce or eliminate side effects due to systemic administration of the agents, particularly over prolonged time periods.

SUMMARY OF THE INVENTION

Biocompatible polymeric materials releasing or producing physiological amounts of nitric oxide (NO) for prolonged periods of time are described herein. The biocompatible polymeric materials are applied to sites on or in a patient in need of treatment thereof for disorders such as restenosis, thrombosis, asthma, wound healing, arthritis, penile erectile dysfunction or

other conditions where NO plays a significant role. The polymeric materials can be formed into films, coatings, or microparticles for application to medical devices, such as stents, vascular grafts and catheters. The polymeric materials can also be applied directly to biological tissues and can be polymerized *in situ*.

The polymers are formed of macromers, which may include biodegradable regions, and have bound thereto groups that are released *in situ* to elevate or otherwise modulate NO levels at the site where treatment is needed. The macromers can form a homo or hetero-dispersion or solution, which is polymerized to form a polymeric material, that in the latter case can be a semi-interpenetrating network or interpenetrating network. Compounds to be released can be physically entrapped, covalently or ionically bound to macromer, or actually form a part of the polymeric material. Hydrogels can be formed by ionic and/or covalent crosslinking. Other active agents, including therapeutic, prophylactic, or diagnostic agents, can also be included within the polymeric material.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a schematic of the synthesis of S-nitrosocysteine hydrogels (Acryloyl-PEG-Cys-NO).

Figure 2 is a schematic of the synthesis of acryloyl-PEG-Lysine$_5$ NO-nucleophile complex hydrogels.

Figure 3 is a schematic of the synthesis of acryloyl-PEG-DETA-NO-nucleophile complex hydrogels.

Figure 4 is a graph showing the temporal release (%NO released over time in days) of NO from acryloyl-PEG-Lys$_5$-NO hydrogels at pH 7.4 (circles) and pH 3 (squares).

Figure 5 is a graph showing the temporal release (%NO released over time in hours) of NO from acryloyl-PEG-DETA-NO hydrogels at pH 7.4 (circles) and pH 2 (squares).

Figure 6 is a graph showing the temporal release (%NO released over time in hours) of NO from PEG-Cys-NO hydrogels at pH 7.4 (circles) and pH 2 (squares).

Figure 7A is a graph showing the temporal release (μmol NO released per gram of polymer over time in hours) of NO from PVA-NO-bFGF hydrogels at pH 7.4, 37°C. Figure 7B is a graph showing the temporal release (% of theoretical bFGF released per gram of gel over time in hours) from PVA-NO-bFGF hydrogels at pH 7.4, 37°C.

Figures 8A and 8B are graphs showing that acryloyl-PEG-Lysine-NO hydrogels inhibit the proliferation of smooth muscle cells. Figure 8A, % of control cell number, hydrogel formulation. Figure 8B, % of control cell number, soluble polymer.

Figures 9A and 9B are graphs showing the inhibition of SMC proliferation by NO released from acryloyl-PEG-DETA-NO hydrogels (Figure 9A) and soluble polymer (Figure 9B), as a percentage of the control.

Figures 10A and 10B are graphs showing inhibition of SMC proliferation by NO released from acryloyl-PEG-Cys-NO hydrogels (Figure 10A) and soluble polymer (Figure 10B), as a percentage of controls.

Figure 11 is a graph comparing the degree of inhibition of smooth muscle cell growth by NO released from hydrogels: acryloyl-PEG-Lys-NO, acryloyl-PEG-DETA-NO, and acryloyl-PEG-Cys-NO, compared to control hydrogel with NO. The percent inhibition of smooth muscle cell growth is determined by comparing the cell growth for each NO-releasing hydrogel to a control PEG-diacrylate hydrogel.

Figure 12 is a graph of endothelial cell proliferation, which was stimulated when cultured in the presence of NO-releasing PEG hydrogels with

varying NO release kinetics. PEG-diacrylate hydrogels were used as a control. The last three bars represent a statistical variance of $p \leq 0.01$ compared to control.

Figure 13 is a graph of endothelial cell proliferation, which was stimulated when cultured on NO-releasing hydrogels that contained the cell adhesion peptide sequence RGDS. The last bar represents a statistical variance of $p < 0.02$ versus either RGDS or DETA-NO hydrogels alone.

Figure 14 is a graph of endothelial cell proliferation, which was stimulated when cultured on NO-releasing hydrogels that contained the cell adhesion peptide sequence REDV. The last bar represents a statistical variance of $p < 0.02$ versus either REDV or DETA-NO hydrogels alone.

Figure 15 is a graph of the proliferation of HDFs cultured in the presence of NO-releasing PVA hydrogels by cell counts. No significant changes in proliferation were observed with exposure to PVA-NO materials.

Figure 16A and 16B are graphs of the matrix production by fibroblasts cultured in the presence of NO-releasing PVA hydrogels. 16A shows the release of NO from PVA hydrogels increased the production of collagen by HDFs ($p < 0.01$ versus control) while 16B shows only slightly increased total matrix produced per cell ($p > 0.05$).

Figure 17A and 17B are graphs comparing wound area and perimeter over time. At the time of each dressing change, pictures of the wounds were taken to assess wound area (17A) and wound perimeter (17B) using image analysis software. No difference in wound area or perimeter was observed between control and NO hydrogel treatment groups.

Figure 18 is a graph of the granulation tissue thickness of wounds by examination of histological sections from wounds treated with PVA or PVA-NO hydrogels. A trend of increasing granulation tissue thickness with increasing NO concentration was observed.

Figure 19 is a graph reflecting wound collagen synthesis. Histological sections from animals at day 29 revealed that wound collagen synthesis was significantly increased through treatment with NO-releasing hydrogels. The second bar represents a statistical variance of $p < 0.001$ versus control.

DETAILED DESCRIPTION OF THE INVENTION

Biocompatible polymeric materials releasing or producing physiological amounts of nitric oxide (NO) and methods of use for the treatment of disorders such as restenosis, thrombosis, asthma, wound healing, arthritis, penile erectile dysfunction, or other conditions where NO plays a significant role, are provided herein.

I. POLYMERIC MATERIALS FOR RELEASE OF NO

The polymeric materials are biocompatible and release or produce NO. In various preferred embodiments, the polymers are also biodegradable, form hydrogels, polymerize *in situ* and are tissue adherent. The polymeric materials can also be formed into films, coatings, or microparticles for application to medical devices, such as stents, vascular grafts and catheters. These properties are conferred by the selection of the macromer components as well as addition of various groups to the components.

The term "polymerizable" means that the regions have the capacity to form additional covalent bonds resulting in macromer interlinking, for example, carbon-carbon double bonds of acrylate-type molecules. Such polymerization is characteristically initiated by free-radical formation resulting from photon absorption of certain dyes and chemical compounds to ultimately produce free-radicals, although polymerization can be obtained using other methods and reagents known to those skilled in the art.

All chemicals mentioned herein are obtainable from commercial chemical companies such as Sigma-Aldrich Chemical Corp. (St. Louis, MO),

unless otherwise specified.

A. Polymeric Materials

The polymeric materials described herein must be biocompatible, i.e., not eliciting a significant or unacceptable toxic or immunogenic response following administration to or implantation into an individual.

A number of polymeric materials are known which are biocompatible, including both natural and synthetic polymers. Examples include proteins (of the same origin as the recipient), polysaccharides such as chondroitin sulfate and hyaluronic acid, polyurethanes, polyesters, polyamides, and acrylates. Polymers can be degradable or non-degradable.

The preferred polymeric materials will be selected based on a combination of properties conferred by the various components, which may include at least one water soluble region, such as polyethylene glycol (PEG) or polyvinyl alcohol (PVA), at least one biodegradable region such as regions that degrade hydrolytically, and at least one group that can be used to polymerize the macromers *in situ*.

One advantage to using the hydrogels described herein is the ability to covalently attach a variety of bioactive molecules. As demonstrated by the stimulation of proliferation of endothelial cells cultured on hydrogels containing both a cell adhesion peptide sequence and an NO donor (Fig. 13), many factors can be combined within the same hydrogel in order to design a material that will perform optimally for the desired application. Examples of cell adhesion peptide sequences (also referred to herein as cell adhesion ligands) include RGD, RGDS, REDV (the letters indicate the single letter amino acid nomenclature known to those skilled in the art), and other sequences that are endothelial cell-specific. The cell adhesion ligands are used to specifically target the adhesion, proliferation, and migration of certain cells. The cell adhesion ligand may be a peptide, protein, carbohydrate, or

other type of moiety that will assist in seeding cells onto devices. For example, other bioactive molecules such as growth factors have also been shown to retain their efficacy when covalently attached to PEG (Mann B, Schmedlen R, West J. Tethered-TGF-beta increases extracellular matrix production of vascular smooth muscle cells. *Biomaterials* 2001; 22: 439-444). Thus, there exists the possibility of creating a multifunctional material that combines NO therapy with at least one cell-specific adhesion ligand or growth factor in order to achieve specific results.

Water-Soluble and/or Tissue Adhesive Regions

A variety of water soluble materials can be incorporated into the polymers. The term "at least substantially water soluble" is indicative that the solubility should be at least about 5 g/100 ml of aqueous solution. In preferred embodiments, the core water soluble region can consist of poly(ethylene glycol), referred to herein as "PEG", poly(ethylene oxide), poly(vinyl acetate), poly(vinyl alcohol), referred to herein as "PVA", poly(vinylpyrrolidone), poly(ethyloxazoline), poly(ethylene oxide)-co-poly(propyleneoxide) block copolymers, polysaccharides or carbohydrates such as hyaluronic acid, dextran, heparin sulfate, chondroitin sulfate, heparin, or alginate, or proteins such as gelatin, collagen, albumin, or ovalbumin.

Hydrophilic (i.e., water soluble) regions will generally be tissue adhesive. Both hydrophobic and hydrophilic polymers which include a large number of exposed carboxylic groups are tissue adhesive or bioadhesive. Ligands such as RGD peptides and lectins which bind to carbohydrate molecules on cells can also be bound to the polymer to increase tissue adhesiveness.

Degradable Regions

Polyesters (Holland *et al.*, 1986 *Controlled Release*, 4:155-180) of α-hydroxy acids (*viz.*, lactic acid, glycolic acid), are the most widely used

biodegradable materials for applications ranging from closure devices (sutures and staples) to drug delivery systems (U.S. Patent No. 4,741,337 to Smith *et al.*; Spilizewski *et al.*, 1985 *J. Control. Rel.* 2:197-203). In addition to the poly(hydroxy acids), several other polymers are known to biodegrade, including polyanhydrides and polyorthoesters, which take advantage of labile backbone linkages, as reported by Domb *et al.*, 1989 *Macromolecules*, 22:3200; Heller *et al.*, 1990 *Biodegradable Polymers as Drug Delivery Systems*, Chasin, M. and Langer, R., Eds., Dekker, New York, 121-161. Polyaminoacids have also been synthesized since it is desirable to have polymers that degrade into naturally occurring materials for *in vivo* use.

The time required for a polymer to degrade can be tailored by selecting appropriate monomers. Differences in crystallinity also alter degradation rates. Due to the relatively hydrophobic nature of these polymers, actual mass loss only begins when the oligomeric fragments are small enough to be water soluble. Hence, initial polymer molecular weight influences the degradation rate.

The biodegradable region is preferably hydrolyzable under *in vivo* conditions. Hydrolyzable groups may be polymers and oligomers of glycolide, lactide, ε-caprolactone, other α-hydroxy acids, and other biologically degradable polymers that yield materials that are non-toxic or present as normal metabolites in the body. Preferred poly(α-hydroxy acid)s are poly(glycolic acid), poly(DL-lactic acid) and poly(L-lactic acid). Other useful materials include poly(amino acids), poly(anhydrides), poly(orthoesters), and poly(phosphoesters). Polylactones such as poly(ε-caprolactone), poly(ε-caprolactone), poly(δ-valerolactone) and poly(gamma-butyrolactone), for example, are also useful.

Biodegradable regions can also be constructed from polymers or monomers using linkages susceptible to biodegradation by enzymes, such as ester, peptide, anhydride, orthoester, and phosphoester bonds. Degradable

materials of biological origin are well known, for example, crosslinked gelatin. Hyaluronic acid has been crosslinked and used as a degradable swelling polymer for biomedical applications (U.S. Patent No. 4,987,744 to della Valle et al., U.S. Patent 4,957,744 to Della Valle *et al.* (1991) *Polym. Mater. Sci. Eng.*, 62:731-735).

Biodegradable Hydrogels

A number of polymers have been described which include both water soluble regions and biodegradable regions. Sawhney et al., (1990) *J. Biomed. Mater. Res.* 24:1397-1411, copolymerized lactide, glycolide and ε-caprolactone with PEG to increase its hydrophilicity and degradation rate. U.S. Patent No. 4,716,203 to Casey et al. (1987) synthesized a PGA-PEG-PGA block copolymer, with PEG content ranging from 5-25% by mass. U.S. Patent No. 4,716,203 to Casey et al. (1987) also reports synthesis of PGA-PEG diblock copolymers, again with PEG ranging from 5-25%. U.S. Patent No. 4,526,938 to Churchill et al. (1985) described noncrosslinked materials with MW in excess of 5,000, based on similar compositions with PEG; although these materials are not water soluble. Cohn et al. (1988) *J. Biomed. Mater. Res.* 22:993-1009 described PLA-PEG copolymers that swell in water up to 60%; these polymers also are not soluble in water, and are not crosslinked. The features that are common to these materials are that they use both water-soluble polymers and degradable polymers, and that they are insoluble in water, collectively swelling up to about 60%.

U.S. Patent No. 5,410,016 issued on April 25, 1995 to Hubbell, et al., describes materials which are based on polyethylene glycol (PEG), because of its high biocompatible and thromboresistant nature, with short polylactide extensions to impart biodegradation and acrylate termini to allow rapid photopolymerization without observable heat production. These materials are readily modified to produce hydrogels which release or produce NO.

The polymerizable regions are separated by at least one degradable region to facilitate uniform degradation *in vivo*. There are several variations of these polymers. For example, the polymerizable regions can be attached directly to degradable extensions or indirectly via water soluble nondegradable sections so long as the polymerizable regions are separated by a degradable section. For example, if the macromer composition contains a simple water soluble region coupled to a degradable region, one polymerizable region may be attached to the water soluble region and the other attached to the degradable extension or region. In another embodiment, the water soluble region forms the central core of the macromer composition and has at least two degradable regions attached to the core. At least two polymerizable regions are attached to the degradable regions so that, upon degradation, the polymerizable regions, particularly in the polymerized gel form, are separated. Conversely, if the central core of the macromer composition is formed by a degradable region, at least two water soluble regions can be attached to the core and polymerizable regions can be attached to each water soluble region. The net result will be the same after gel formation and exposure to *in vivo* degradation conditions.

In another embodiment, the macromer composition has a water soluble backbone region and a degradable region affixed to the macromer backbone. At least two polymerizable regions are attached to the degradable regions, so that they are separated upon degradation, resulting in gel product dissolution. In a further embodiment, the macromer backbone is formed of a nondegradable backbone having water soluble regions as branches or grafts attached to the degradable backbone. Two or more polymerizable regions are attached to the water soluble branches or grafts. In another variation, the backbone may be star shaped, which may include a water soluble region, a biodegradable region or a water soluble region which is also biodegradable. In this general embodiment, the star region contains either water soluble or

biodegradable branches or grafts with polymerizable regions attached thereto. Again, the polymerizable regions must be separated at some point by a degradable region.

Polymerizable groups.

The polymerizable regions may be polymerizable by photoinitiation by free radical generation, most preferably in the visible or long wavelength ultraviolet radiation. The preferred polymerizable regions are acrylates, diacrylates, oligoacrylates, dimethacrylates, oligomethoacrylates, or other biologically acceptable photopolymerizable groups. A preferred tertiary amine is triethanol amine.

Useful photoinitiators are those which can be used to initiate by free radical generation polymerization of the macromers without cytotoxicity and within a short time frame, minutes at most and most preferably seconds. Preferred dyes as initiators of choice for long wavelength ultraviolet (LWUV) light initiation are ethyl eosin, 2,2-dimethoxy-2-phenyl acetophenone, other acetophenone derivatives, other eosin derivatives, such as eosin Y, and camphorquinone. In all cases, crosslinking and polymerization are initiated among copolymers by a light-activated free-radical polymerization initiator such as 2,2-dimethoxy-2-phenylacetophenone or a combination of ethyl eosin (10^{-4}-10^{-2} mM) and triethanolamine (0.001 to 0.1 M), for example.

The choice of the photoinitiator is largely dependent on the photopolymerizable regions. For example, when the macromer includes at least one carbon-carbon double bond, light absorption by the dye causes the dye to assume a triplet state, the triplet state subsequently reacting with the amine to form a free radical which initiates polymerization. Preferred dyes for use with these materials include eosin dye and initiators such as 2,2-dimethyl-2-phenylacetophenone, 2-methoxy-2-phenylacetophenone, and camphorquinone. Using such initiators, copolymers may be polymerized *in*

situ by long wavelength ultraviolet light or by laser light of about 514 nm, for example.

Initiation of polymerization is accomplished by irradiation with light at a wavelength of between about 200-700 nm, most preferably in the long wavelength ultraviolet range or visible range, 320 nm or higher, most preferably about 514 nm or 365 nm.

There are several photooxidizable and photoreducible dyes that may be used to initiate polymerization. These include acridine dyes, for example, acriblarine; thiazine dyes, for example, thionine; xanthine dyes, for example, rose bengal; and phenazine dyes, for example, methylene blue. These are used with cocatalysts such as amines, for example, triethanolamine; sulphur compounds, for example, RSO_2R_1; heterocycles, for example, imidazole; enolates; organometallics; and other compounds, such as N-phenyl glycine. Other initiators include camphorquinones and acetophenone derivatives.

Thermal polymerization initiator systems may also be used. Such systems that are unstable at 37°C and would initiate free radical polymerization at physiological temperatures include, for example, potassium persulfate, with or without tetramethyl ethylenediamine; benzoylperoxide, with or without triethanolamine; and ammonium persulfate with sodium bisulfite.

Other initiation chemistries may be used besides photoinitiation. These include, for example, water and amine initiation schemes with isocyanate or isothiocyanate containing macromers used as the polymerizable regions.

Preferred Embodiments

In a preferred embodiment, the polymeric materials in the macromer composition are polymerizable and at least substantially water soluble. A first macromer includes at least one water soluble region, at least one NO carrying region, and at least one free radical-polymerizable region. A second macromer

includes at least one water soluble region and at least two free radical polymerizable regions. The regions can, in some embodiments, be both water soluble and biodegradable. The macromer composition is polymerized by exposure of the polymerizable regions to free radicals generated, for example, by photosensitive chemicals and dyes.

Examples of these macromers are PVA or PEG. The choice of appropriate end caps permits rapid polymerization and gelation. Acrylates are preferred because they can be polymerized using several initiating systems, e.g., an eosin dye, by brief exposure to ultraviolet or visible light. A PEG central structural unit (core) is preferred on the basis of its high hydrophilicity and water solubility, accompanied by excellent biocompatibility. A short oligo or poly(α-hydroxy acid), such as polyglycolic acid, can be used as a biodegradable chain extension because it rapidly degrades by hydrolysis of the ester linkage into glycolic acid, a harmless metabolite. Although highly crystalline polyglycolic acid is insoluble in water and most common organic solvents, the entire macromer composition is water-soluble and can be rapidly gelled into a biodegradable network while in contact with aqueous tissue fluids. Such networks can be used to entrap and homogeneously disperse water-soluble drugs and enzymes and to deliver them at a controlled rate. Further, they may be used to entrap particulate suspensions of water-insoluble drugs. Other preferred chain extensions are polylactic acid, polycaprolactone, polyorthoesters, and polyanhydrides. Polypeptides may also be used. Such "polymeric" blocks should be understood to included dimeric, trimeric, and oligomeric blocks.

PVA contains many pendant hydroxyl groups. These hydroxyl groups are easily reacted to form side chains such as various crosslinking agents and nitric oxide donors. PVA is water soluble and has excellent biocompatiblity. Modification of PVA to attach methacrylate groups via a diacetal bond with the pendant hydroxyl groups and addition of an appropriate photoinitiator

enables the PVA to be photopolymerized to form hydrogels under long wavelength UV light. In another preferred embodiment, the hydrogel is formed from modified polyvinyl alcohol (PVA) macromers, such as those described in U.S. Patent Nos. 5,508,317, 5,665,840, 5,849,841, 5,932,674, 6,011,077, 5,939,489, and 5,807,927. The macromers disclosed in U.S. Patent No. 5,508,317, for example, are PVA prepolymers modified with pendant crosslinkable groups, such as acrylamide groups containing crosslinkable olefinically unsaturated groups. These macromers can be polymerized by photopolymerization or redox free radical polymerization, for example. Several embodiments of the macromers of the invention are disclosed herein describing formulations for photopolymerizable macromers. However, one of skill in the art, after studying this disclosure, would know how to make and use macromers formulated for other methods of polymerization. The starting polymers are, in particular, derivatives of polyvinyl alcohol or copolymers of vinyl alcohol that contain, for example, a 1,3-diol skeleton. The crosslinkable group or the further modifier can be bonded to the starting polymer skeleton in various ways, for example through a certain percentage of the 1,3-diol units being modified to give a 1,3-dioxane, which contains a crosslinkable radical, or a further modifier in the 2-position. Another possibility is for a certain percentage of hydroxyl groups in the starting polymer to be esterified by means of an unsaturated organic acid, these ester-bonded radicals containing a crosslinkable group. The hydrophobicity of these macromers can be increased by substituting some of the pendant hydroxyl groups with more hydrophobic substituents. The properties of the macromers, such as hydrophobicity, can also be modified by incorporating a co-monomer in the macromer backbone. The macromers can also be formed having pendant groups crosslinkable by other means.

B. NO groups or Modulating Compounds

A number of molecules that produce NO under physiological conditions (NO donors) have been identified and evaluated both *in vitro* and *in vivo,* including S-nitrosothiols, organic nitrates, and complexes of NO with nucleophiles. L-arginine is a NO donor, since L-arginine is a substrate for NO synthase, and thus administration of L-arginine increases endogenous NO production and elicits responses similar to those caused by NO donors in most cases. Other NO donors include molsidomine, CAS754, SPM-5185, and SIN-1. Other compounds capable of producing and/or donating NO may also be used. These include organic nitrates, nitrosylating compounds, nitrosoesters, and L-arginine.

The molecules which produce NO, or release or generate NO, are preferably attached to regions containing nucleophiles and/or thiols such as S-nitrosothiols capable of forming a complex with NO.

C. Prophylactic, Therapeutic and Diagnostic Agents

The polymeric materials can also be used for drug delivery, preferably localized release of prophylactic, therapeutic or diagnostic agents at the site where the materials are needed, although the polymeric materials can be loaded with agent to be released systemically. These agents include proteins or peptides, polysaccharides, nucleic acid molecules, and simple organic molecules, both natural and synthetic. Representative materials include antibiotics, antivirals, and antifungal drugs, anti-inflammatories (steroidal or non-steroidal), hormones, growth factors, cytokines, neuroactive agents, vasoconstrictors and other molecules involved in the cardiovascular responses, enzymes, antineoplastic agents, local anesthetics, antiangiogenic agents, antibodies, drugs affecting reproductive organs, and oligonucleotides such as antisense oligonucleotides. Diagnostic materials may be radioactive, bound to or cleave a chromogenic substrate, or detectable by ultrasound, x-ray, MRI, or other standard imaging means.

These agents can be mixed with macromer prior to polymerization, applied into or onto the polymer, or bound to the macromer prior to or at the time of polymerization, either covalently or ionically, so that the agent is released by degradation (enzymatic or hydrolytic) or diffusion at the site where the polymer is applied.

II. Methods of Use

A. Coatings; Films; Microparticles

Although described primarily with respect to *in vivo* treatment, it is apparent that the polymeric materials described herein can be used in cell culture, on cell culture substrates, or as coatings on medical implants or devices such as stents, vascular grafts, or catheters, or formed using standard techniques into microparticles or other types of formulations which may be used in or administered to a patient.

For example, coatings on medical devices or implants may be used when the proliferation of endothelial cells over the medical device would prolong the use and safety of the device. One of skill in the art may envision many such uses. An example is found in dialysis. The coating would allow endothelial proliferation over the graft used during a dialysis session, thereby prolonging the usable time for each graft, thus delaying the need for a kidney transplant. Coatings may provide other advantages seen upon local delivery of NO such as decreased proliferation of smooth muscle cells and decreased platelet aggregation.

B. Therapeutic Applications

Polymeric materials capable of releasing physiological amounts of NO for prolonged periods of time can be applied to sites on or in a patient in need of treatment thereof. Representative disorders or conditions that can be treated with NO include restenosis, thrombosis, asthma, wound healing, arthritis, and penile or female erectile dysfunction. The material can be applied as a macromer solution and polymerized *in situ* or polymerization can

be initiated prior to application. The polymeric materials can also be coated onto medical devices.

Wound Healing

The formulations are particularly useful for treatment of all types of wounds, including burns, surgical wounds, and open leg and foot wounds. There are generally three types of open leg wounds, termed ulcers: venous stasis ulcers, generally seen in sedentary elderly people when blood flow to the leg becomes sluggish; decubitus ulcers, also termed pressure sores or bed sores, which occurs most often in people who are bedridden and are unable to frequently change position; and diabetic foot ulcers, caused by poor blood circulation to the feet. Due to the aging of the population, there will likely be a greater demand for effective and user friendly wound treatments in the near future.

The term "wound" as used herein refers to all types of tissue injuries, including those inflicted by surgery and trauma, including burns, as well as injuries from chronic or acute medical conditions, such as atherosclerosis or diabetes.

Example 13 shows that exogenous NO released from hydrogel wound dressings may enhance wound healing of chronic wounds. *In vivo* results examining effects of NO in the diabetic wound model suggest that the most useful parameters for assessing efficacy of wound healing are granulation tissue thickness and matrix production. Similar findings have been reported in studies examining the effect of growth factors on wound healing in animals (Greenhalgh D. The role of growth factors, in wound healing. *J Trauma* 1996; 41: 159-167). A review of multiple animal models for assessing efficacy of PDGF concluded that epithelialization and wound contraction were not significantly altered, whereas in most models, including the diabetic mouse model, granulation tissue thickness was consistently increased following application of the growth factor (LeGrand EK. Preclinical promise of

Becaplermin (rhPDGF-BB) in wound healing. *Am J Surg* 1998; 176:48S-54S).

Similar improvements in wound healing appear to occur following application of NO, and this might be attributed to the inter-related mechanisms of action of NO with growth factors.

The significant increase in *in vivo* wound collagen deposition caused by treatment with NO indicates that delivery of NO from PVA hydrogel wound dressings may lead to the development of a more structurally stable closed wound. This finding is important, as chronic wounds are frequently complicated by their inability to remain healed due to insufficient mechanical integrity.

NO clearly plays a critical role in the wound healing process, most probably via multiple mechanisms including increased cell proliferation via upregulation of growth factor receptors and upregulation of matrix synthesis. That growth factors alone also enhance wound healing suggests that combining NO with growth factors may lead to synergistic effects.

Hydrogels may be modified to covalently attach growth factors, while maintaining the bioactivity of the growth factor (Mann B, Schmedlen R, West J. Tethered-TGF-beta increases extracellular matrix production of vascular smooth muscle cells. *Biomaterials* 2001; 22: 439-444). Thus, it is possible to develop hydrogels that provide combined NO and growth factor therapy to further enhance the healing of chronic wounds.

The materials described herein overcome some of the disadvantages of existing wound treatments by allowing the formation of hydrogel coatings through *in situ* polymerization. This technology may simplify the often difficult application of wound dressings to areas such as foot ulcers. Additionally, a multitude of factors to promote wound healing may be incorporated into these dressings through simple modification of the hydrogel material.

Treatment of Restenosis

A preferred application is a method of reducing the effects of restenosis on post-surgical patients. One embodiment of the method includes coating the surface within an artery with an aqueous solution of light-sensitive free radical polymerizable initiator and a number of macromers. The coated artery is subjected to a Xenon arc laser inducing polymerization of the macromers. As the newly polymerized macromer composition is formed, the physiological conditions within the artery will induce the release of NO. This release will be strictly localized for prolonged periods of time. In another embodiment of the method, a stent coated with the NO-releasing hydrogel is implanted in an artery.

Prevention of Surgical Adhesions.

A preferred application is a method of reducing formation of adhesions after a surgical procedure in a patient. In one embodiment the method includes coating damaged tissue surfaces in a patient with an aqueous solution of a light-sensitive free-radical polymerization initiator and a macromer solution as described above. The coated tissue surfaces are exposed to light sufficient to polymerize the macromer. The light-sensitive free-radical polymerization initiator may be a single compound (e.g., 2,2-dimethoxy-2-phenyl acetophenone) or a combination of a dye and a cocatalyst (e.g., ethyl eosin and triethanol amine).

Tissue Adhesives.

Another use of the polymers is in a method for adhering tissue surfaces in a patient. In one embodiment the macromer is mixed with a photoinitiator or photoinitiator/cocatalyst mixture to form an aqueous mixture and the mixture is applied to a tissue surface to which tissue adhesion is desired. The tissue surface is contacted with the tissue with which adhesion is desired, forming a tissue junction. The tissue junction is then irradiated until the macromers are polymerized.

Tissue Coatings.

In a particularly preferred application of these macromers, an ultrathin coating is applied to the surface of a tissue, most preferably the lumen of a tissue such as a blood vessel. One use of such a coating is in the treatment or prevention of restenosis, abrupt reclosure, or vasospasm after vascular intervention. An initiator is applied to the surface of the tissue, allowed to react, adsorb or bond to tissue, the unbound initiator is removed by dilution or rinsing, and the macromer solution is applied and polymerized. This method is capable of creating uniform polymeric coating of between one and 500 microns in thickness, most preferably about twenty microns, which does not evoke thrombosis or localized inflammation.

Tissue Supports.

The polymeric materials can also be used to create tissue supports by forming shaped articles within the body to serve a mechanical function. Such supports include, for example, sealants for bleeding organs, sealants for bone defects and space-fillers for vascular aneurisms. Further, such supports can include strictures to hold organs, vessels or tubes in a particular position for a controlled period of time.

Controlled drug delivery.

As noted above, the polymeric materials can be use as carriers for biologically active materials such as therapeutic, prophylactic or diagnostic agents, including hormones, enzymes, antibiotics, antineoplastic agents, and cell suspensions. The polymeric material may be used to temporarily preserve functional properties of an agent to be released, as well as provide prolonged, controlled release of the agent into local tissues or systemic circulation.

In a variation of the method for controlled drug delivery in which an agent is mixed with the macromer solution then polymerized *in situ*, the macromers are polymerized with the biologically active materials to form microspheres or nanoparticles containing the biologically active material. The

macromer, photoinitiator, and agent to be encapsulated are mixed in an aqueous mixture. Particles of the mixture are formed using standard techniques, for example, by mixing in oil to form an emulsion, forming droplets in oil using a nozzle, or forming droplets in air using a nozzle. The suspension or droplets are irradiated with a light suitable for photopolymerization of the macromer.

These materials are particularly useful for controlled drug delivery of hydrophilic materials, since the water soluble regions of the polymer enable access of water to the materials entrapped within the polymer. Moreover, it is possible to polymerize the macromer composition containing the material to be entrapped without exposing the material to organic solvents. Release may occur by diffusion of the material from the polymer prior to degradation and/or by diffusion of the material from the polymer as it degrades, depending upon the characteristic pore sizes within the polymer, which is controlled by the molecular weight between crosslinks and the crosslink density. Deactivation of the entrapped material is reduced due to the immobilizing and protective effect of the gel and catastrophic burst effects associated with other controlled-release systems are avoided. When the entrapped material is an enzyme, the enzyme can be exposed to substrate while the enzyme is entrapped, provided the gel proportions are chosen to allow the substrate to permeate the gel. Degradation of the polymer facilitates eventual controlled release of free macromolecules in vivo by gradual hydrolysis of the terminal ester linkages.

As demonstrated by examples 1-3 below, three classes of NO-producing, PEG-based polymers have been synthesized and their NO release rate constants determined in vitro under physiological conditions. The biological response to appropriate materials has been evaluated in vitro using cultured smooth muscle cells and endothelial cells and in vivo using a rat carotid artery injury model that resembles restenosis in man. The materials

include BAB block copolymers of polyethylene glycol (A) with polycysteine (B) that are subsequently reacted with $NaNO_2$ to form S-nitrosothiols, BAB block copolymers of polyethylene glycol ("PEG") (A) and diethylenetriamine ("DETA") (B) that are subsequently reacted with NO gas to form nucleophile/NO complexes, and BAB block copolymers of polyethylene glycol (A) and polylysine (B) that are subsequently reacted with NO gas to form nucleophile/NO complexes. Blended compounds may also be prepared for providing biphasic release of profiles, such as PEG-Cys-DETA-NO. All polymers are further terminated with reactive acrylate groups to allow rapid polymerization *in situ*.

Such materials would be expected to have good biocompatibility, provided that a water soluble, biocompatible polymer such as PEG comprises the bulk of the material and has a sufficiently high molecular weight, and to slowly biodegrade due to the presence of two ester bonds and two amide bonds in each polymer chain. These three materials were selected as they are expected to have vastly different release kinetics: nucleophile/NO complexes have been shown to release NO for up to 5 weeks (Smith el al., (1996) *J. Med. Chem.*, 39:1148-56), while the half-life of S-nitrosocysteine is 0.023 hours (Mathews et al., (1993) *J. Pharmacol. Exp. Therap.*, 267:1529-37). The amount of NO produced by these copolymers may be tailored by altering the ratio of polyethylene glycol (PEG) to cysteine or lysine.

An advantage of these macromer compositions are that they can be polymerized rapidly in an aqueous surrounding. Precisely conforming, semi-permeable, biodegradable films or membranes can thus be formed on tissue in situ to serve as biodegradable barriers, as carriers for living cells or other biologically active materials, and as surgical adhesives. The polymer shows excellent biocompatibility, as seen by a minimal fibrous overgrowth on implanted samples. Hydrogels for the models were gelled in situ from water-soluble precursors by brief exposure to LWUV light, resulting in formation of

an interpenetrating network of the hydrogel with the protein and glycosaminoglycan components of the tissue.

As demonstrated by examples 4 and 5 below, three types of PVA hydrogels were made and demonstrated release of NO and incorporated drug (bFGF): PVA-NH_2-NO hydrogels; PVA-Cys-NO hydrogels; PVA-NO-bFGF hydrogels. The results are similar to those for the PEG based hydrogels.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are intended neither to limit nor define the invention in any manner.

EXAMPLES

EXAMPLE 1: SYNTHESIS OF PEG-CYS-NO MACROMERS AND HYDROGELS.

As shown in Figure 1, an acryloyl-PEG-Cys-NO polymer was formed by first reacting polyethylene glycol N-hydroxysuccinimide monoacrylate (ACRL-PEG-NHS, MW 3400, commercially available from Shearwater Polymers, Huntington, AL) with L-cysteine at an 1:2 molar ratio in 50 mM sodium bicarbonate buffer (pH 8.5) for 2 hours; the product was then dialyzed in a cellulose ester membrane (Molecular weight cutoff 500, Spectrum Labs, Laguna Hills, CA) in diH_2O, and lyophilized. Analysis of the acryloyl-PEG-Cys copolymer was performed using gel permeation chromatography (GPC) with an evaporative light scattering detector and a UV detector at 260 nm (Polymer Laboratories, Amherst, MA). Successful synthesis of acryloyl-PEG-Cys was determined by a shift in the position of the peak from the evaporative light scattering detector. The copolymer was then reacted with an equimolar amount of $NaNO_2$ at pH 2 and 37°C for 20 minutes to form S-nitrosocysteine. Conversion of thiol groups to S-nitrosothiols was measured using the Ellman's assay (Hermanson, (1995) *Bioconjugate Techniques,* San Diego, CA Academic Press; 88-90). After adjusting the pH of the solution to 7.4, the acryloyl-PEG-Cys-NO polymer was incorporated into

photopolymerizable hydrogels by mixing with PEG-diacrylate (MW 3400) at a 1:10 molar ratio in aqueous solution with 1500 ppm 2,2-dimethoxy-2-phenyl acetophenone as a long wavelength ultraviolet initiator. 0.15% N-vinylpyrrolidone was present in this mixture as it was used as a solvent for the photoinitiator. Exposure to UV light (365 nm, 10 mW/cm^2) was used to crosslink the polymer, resulting in conversion to a hydrogel (Sawhney et al., (1993) *Macromol* 26:581-7).

EXAMPLE 2: SYNTHESIS OF PEG-LYS_5-NO MACROMERS AND HYDROGELS.

As shown in Figure 2, for acryloyl-PEG-Lys_5-NO hydrogels, a copolymer of ACRL-PEG-NHS (MW 3400, Shearwater Polymers) and poly-L-lysine (DP=5) was synthesized by reacting at an equimolar ratio in 50 mM sodium bicarbonate (pH 8.5). The resultant copolymer was analyzed via GPC, then dissolved in water and reacted with NO gas in an evacuated vessel, thus forming NO-nucleophile complexes with the amine groups on the lysine side groups. The extent of conversion of amine groups to NO-nucleophile complexes was measured using the ninhydrin assay, and crosslinked hydrogels were formed as described above in Example 1.

EXAMPLE 3: SYNTHESIS OF PEG-DETA-NO MACROMERS AND HYDROGELS.

Diethylenetriamine (DETA, Aldrich, Milwaukee, WI) was reacted with ACRL-PEG-NHS (MW 3400, Shearwater Polymers) in 50 mM sodium bicarbonate buffer (pH 8.5) at an equimolar ratio, lyophilized, and analyzed via GPC as described above. The copolymer was then dissolved in water and exposed to NO gas to form NO-nucleophile complexes as described for PEG-Lys_5-NO and assayed for amine content using the ninhydrin assay. The PEG-DETA-NO was lyophilized and then photopolymerized as described above to form hydrogels, as shown in Figure 3.

EXAMPLE 4: SYNTHESIS OF PVA-NH_2-NO MACROMERS AND HYDROGELS.

Poly(vinyl alcohol) (Hoechst, Mowiol 4-88) was dissolved in di$H_2$0 and warmed to 95°C in a round bottom flask under continuous stirring. After one hour, the solution was cooled to room temperature, and a crosslinkable acetal group, methacrylamidoacetaldehyde dimethyl acetal (NAAADA) was added. The amine acetal, gamma-aminobutyraldehyde diethyl acetal, was also added, and the mixture was acidified using glacial acetic acid and 37% hydrochloric acid. The mixture was allowed to stir at room temperature for nine hours, after which the pH was adjusted to pH 3.6 using triethylamine. In order to purify the polymer, the solution was then diafiltered through a MW 3000 cellulose membrane against diH_2O at 6.5 times the volume of polymer solution. The polymer concentration was adjusted to 22% w/v using diafiltration, and the pH was adjusted to 7.4 with 1N NaOH. The amine concentration of the polymer was determined using the ninhydrin assay.

In order to form the NO donor bound to the PVA-NH_2, the neutralized amine-modified polymer was placed in a round bottom flask with stopcock. The flask was evacuated and filled with nitric oxide gas until the desired conversion of amines to NO nucleophile complexes was achieved. Photocrosslinked hydrogels were formed from the PVA-NH_2-NO by adding 0.1% IRGACURE™ 2959 (Ciba-Geigy) photoinitiator (based on total solution volume) and then exposing to UV light (2 mW/cm^2, 365 nm) for 30 seconds. Addition of the photoinitiator brings the final polymer concentration to 20% w/v.

EXAMPLE 5: SYNTHESIS OF PVA-CYS-NO MACROMERS AND HYDROGELS.

PVA-NH_2 was synthesized as described above. The amine terminus of cysteine was acetylated using acetic anhydride, and the carboxyl end of the cysteine was coupled to the PVA-NH_2 using water-soluble EDAC chemistry.

The resulting PVA-Cys was then purified using diafiltration and brought to a concentration of 22% w/v. PVA-Cys-NO was formed by adding sodium nitrite at an equimolar amount to cysteine residues, adjusting the pH to 2, and incubating at 37°C for 15 minutes. The extent of reaction of cysteine to Cys-NO was assayed using both the Ellman's and Griess reactions. The photoinitiator, 2,2-methyl-2-phenylacetophenone was dissolved in N-vinylpyrrolidone at a concentration of 600 mg/ml and added to the polymer solution (0.1% based on total solution volume). The polymer was then crosslinked under UV light for 30 seconds and placed in HEPES buffered saline, pH 7.4, 37°C.

EXAMPLE 6: SYNTHESIS OF PVA-NO-BFGF HYDROGELS.

For PVA-NO-bFGF hydrogels, the above procedure was used to make the PVA-NO polymer. Immediately prior to exposure to UV light, 25 μg/ml bFGF was added to the polymer solution and mixed well. Gels were crosslinked as described earlier and stored in HEPES buffered saline, pH 7.4, 37°C.

EXAMPLE 7: NO RELEASE FROM HYDROGELS.

Following preparation and polymerization of the NO-releasing materials as described above, the hydrogels were weighed and stored in HEPES buffered saline, pH 7.4, at 37°C. Aliquots of the buffer were removed at each time point and replaced with fresh buffer. The samples from each time point were then analyzed for nitrite content using a colorimetric assay based on the Griess reaction.

NO release kinetics of hydrogels stored in buffer at various pH levels were also investigated in order to explore possible storage conditions for the hydrogels. At acidic pH levels, release of NO from the hydrogels was significantly inhibited.

NO release from acryloyl-PEG-Lys_5-NO hydrogels is shown in Figure 4.

NO release from acryloyl -PEG-DETA-NO hydrogels is shown in Figure 5.

NO release from acryloyl-PEG-Cys-NO hydrogels is shown in Figure 6.

EXAMPLE 8: NO AND BFGF RELEASE FROM PVA-NO-BFGF HYDROGELS.

The release of NO release from PVA-NO-bFGF hydrogels prepared as described in Example 6 was determined in the same manner as Example 7 and is shown in Figure 7A. Release of bFGF was quantified using that BCA assay (Pierce Chemicals) and is shown in Figure 7B. Release of NO continues for well over 12 hours, while the growth factor is completely released within the first 5 hours.

EXAMPLE 9: EFFECTS OF NO-RELEASING MACROMERS ON CULTURED SMOOTH MUSCLE CELLS: PROLIFERATION AND VIABILITY.

In order to assess the potential of a material for the reduction of smooth muscle cell proliferation after vascular injury, cultured smooth muscle cells were grown in the presence of NO-releasing materials, and the effects of those materials on the cells evaluated. Smooth muscle cells isolated from Wistar-Kyoto rats (passage 11-15, provided by T. Scott-Burden) were cultured in Minimum Essential Medium supplemented with 10% FBS, 2 mM L-glutamine, 500 units penicillin, and 100 mg/L streptomycin, at 37°C in a 5% CO_2 environment. The cells were seeded into 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) at a density of 10,000 cells/cm^2. NO donors in either soluble or hydrogel form were added to the media in the wells one day after seeding. At 4 days culture, cell numbers were determined by preparing single cell suspensions with trypsin and counting three samples from each group using a Coulter counter (Multisizer #0646, Coulter Electronics, Hialeah, FL).

The effects of NO donors in solution on the proliferation of smooth muscle cells were first investigated by performing a NO dose response curve, whereupon cells were cultured with a range of NO donor concentrations (1 μM - 10 mM) in order to identify appropriate dosages for hydrogel studies. NO-nucleophile complexes (Lys-NO and DETA-NO) were formed by reacting either L-lysine or DETA with NO gas in water for 24 hours. Soluble Cys-NO was synthesized by reacting an equimolar amount of L-cysteine with $NaNO_2$ at pH 2 and 37°C for 20 minutes. All NO donor solutions were adjusted to pH 7.4 prior to addition to cell cultures.

Smooth muscle cell proliferation in the presence of NO-producing and control hydrogels was then investigated using the optimal NO dose determined above. Hydrogels containing acryloyl-PEG-Lys_5-NO, acryloyl-PEG-DETA-NO, and acryloyl-PEG-Cys-NO were formed as described above, except that the gel solutions were sterile filtered through 0.2 μm syringe filters (Gelman Sciences, Ann Arbor, MI) prior to adding 2,2-dimethoxy-2-phenyl acetophenone. PEG-diacrylate hydrogels containing no NO donors were used as a control. The hydrogels were photopolymerized in cell culture inserts (8 μm pore size, Becton Dickinson, Franklin Lakes, NJ) and placed in the media over the cultured cells.

All three hydrogel NO donors significantly inhibited SMC growth ($p < 0.0001$). The number of smooth muscle cells remained near that of the seeding density, which ranged from 10-15% of the final control cell number for all experiments.

Inhibition of SMC proliferation by acryloyl-PEG-Lys_5-NO hydrogels is shown in Figure 8A, compared to the macromer solution control shown in Figure 8B. Both significantly inhibited SMC proliferation.

Inhibition of SMC proliferation by acryloyl-PEG-DETA-NO-nucleophile complex hydrogels is shown in Figure 9A, compared to the macromer solution control shown in Figure 9B. Both significantly inhibited

SMC proliferation.

Inhibition of SMC proliferation by acryloyl-PEG-Cys-NO hydrogels is shown in Figure 10A, compared to the macromer solution control shown in Figure 10B. Both significantly inhibited SMC proliferation.

Inhibition of SMC proliferation by acryloyl-PEG-Cys-NO hydrogels, acryloyl-PEG-DETA-NO hydrogels, and acryloyl-PEG-Lys-NO hydrogels is compared to the control hydrogel in Figure 11. All of the NO hydrogels significantly inhibited SMC growth.

EXAMPLE 10: EFFECTS OF NO-RELEASING MACROMERS ON PLATELET ADHESION *IN VITRO*.

The effect of NO release on platelet adhesion was investigated to assess the potential of these materials for prevention of thrombosis. Blood was obtained from a healthy volunteer by venipuncture and anticoagulated with 10 U/ml heparin. Platelets and white blood cells were fluorescently labeled with mepacrine at a concentration of 10 μM. A solution of 2.5 mg/ml collagen I in 3% glacial acetic acid in diH_2O was prepared and applied to glass slides for 45 minutes in a humidified environment at room temperature. Acryloyl-PEG-Cys-NO and PEG-diacrylate hydrogels were prepared as described above and incubated with the labeled whole blood at 37°C for 30 minutes. The hydrogels were removed and the blood was then incubated with the collagen-coated glass slides (two per group) for 20 minutes at 37°C and then rinsed with HBS. Platelet counts per field of view at 40x were counted under a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY) in four randomly chosen areas per slide.

Photos of platelets which had been exposed to control PEG-diacrylate or acryloyl-PEG-Cys-NO hydrogels demonstrate that exposure to the NO-releasing hydrogels inhibits platelet adhesion to thrombogenic surfaces. Glass slides coated with collagen were used as a thrombogenic surface to which platelets would normally adhere. When the blood was incubated with control